

## Thr226 Is a Key Residue for Bioluminescence Spectra Determination in Beetle Luciferases

Vadim Viviani,\*†<sup>1</sup> A. Uchida,\* N. Suenaga,\* M. Ryufuku,\* and Y. Ohmiya\*‡

\*Department of Biochemistry, Shizuoka University, Shizuoka-shi, Japan; †Molecular and Cell Biology, Harvard University, Cambridge, Massachusetts 02138; and ‡Department of Organic Materials, Osaka National Research Institute, Ikeda, Osaka, Japan

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The comparison of click beetle and railroadworm luciferases (pH-insensitive) with firefly luciferases (pH-sensitive) showed a set of conserved residues differing between the two groups which could be involved with the bioluminescence spectra pH sensitivity. The substitution C258V in *Pyrocoelia miyako* (Pml) firefly luciferase and V255C in *Ragophthalmus ohbai* railroad worm luciferase (Rol) had no effect on the bioluminescence spectra. Substitution of Thr226 in the green-light-emitting luciferases of Rol and *Pyrearinus termitilluminans* (Pyt) click beetle luciferases resulted in red-shifts (12 to 35 nm), whereas the substitution T226N in the red-light-emitting luciferase of *Phrixothrix hirtus* (PhRE) railroadworm resulted in a 10 nm blue-shift. In Pml the substitution N230S resulted in a typical red mutant ( $\lambda_{\max}$  = 611 nm). The bioluminescence spectrum of all these luciferase mutants did not show altered pH-sensitivity nor considerably changed half-bandwidth in relation to the wild-type luciferases. Altogether present data suggest that Thr226 is an important residue for keeping active-site core in both groups of beetle luciferases. The mechanism for bioluminescence color determination between pH-sensitive and pH-insensitive luciferases could be different. © 2001 Academic Press

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Bioluminescence in beetles is characterized by a wide range of colors: green–yellow in fireflies (1), green–orange in click-beetles (2), and green–red in

Abbreviations used: EDTA, ethylenediamine-*N,N,N'*-tetraacetic acid; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; DTT, dithiothreitol; CoA, coenzyme A; BL, bioluminescence; LB, Luria-Bertani medium; PhRe, *Phrixothrix hirtus* red; PvGr, *Phrixothrix vivianii* green; Rol, *Ragophthalmus ohbai*; Pyt, *Pyrearinus termitilluminans*; Pml, *Pyrocoelia miyako*.

<sup>1</sup>To whom correspondence should be addressed at Molecular and Cell Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138. Fax: (617) 495-8557. E-mail: [viviani@fas.harvard.edu](mailto:viviani@fas.harvard.edu).

railroad worms (3). This variety of bioluminescence colors was attributed to the luciferase structures, since luciferin is the same in all forms (1–3). Three mechanisms have been proposed to determine bioluminescence color in the active-site of beetle luciferases (4): (i) the polarity of the active-site (5); (ii) presence of basic residues assisting oxyluciferin tautomerization (6); and (iii) the active-site conformation, which may govern the degree of rotation of oxyluciferin thiazoline rings (7). Knowledge of the primary structure of many beetle luciferases (8–18), mostly from fireflies, and mutagenesis studies (19–24) have shown that the region around residues 209–300 in firefly luciferases and 220–247 in click beetle luciferases determines the bioluminescence color. In addition, several single sites along the region 300–452 in firefly luciferases were shown to affect dramatically the bioluminescence color (20, 22, 23). The crystallographic structure of firefly luciferase was solved in the absence of bound substrates (25), showing a large N-terminal domain (residues 1–436) and a small C-terminal domain (residues 440–550) which form a cleft containing many conserved residues in their surfaces. Based on conserved residues and mutagenesis studies, active-site models of firefly luciferase were proposed (24, 26), however, definitive determination of active site structure awaits confirmation by crystallography with bound substrates. Although there are many studies focusing firefly luciferases, much less study was done in order to compare the structure–function relationship in click beetles and mainly railroad worm luciferases.

The bioluminescence spectra of firefly luciferases (pH-sensitive) have been long shown to undergo red shift at lower pHs and other denaturant conditions (27), whereas click beetle and railroad worm luciferases (pH-insensitive) were systematically shown to be insensitive to the same conditions (28). The comparison of railroadworm and click beetle luciferases primary structures with a large set of firefly luciferases have shown conserved residues within each group of

luciferases, but which differ between the two groups, which could be involved with the pH-sensitivity (17). We have started a site-directed mutagenesis study in order to determine the effect of the substitution of these residues on the bioluminescence spectra and their pH-sensitivity. Previously, we have shown that the region before residue 344 contains the bioluminescence color determinant in *Phrixothrix* railroadworm luciferases and Arg 215 is an important residue for bioluminescence color determination (29). Here we report the effect of the substitution of Val255 (Val in pH-insensitive luciferases and Cys in pH-sensitive luciferases) and Thr226 (Thr in pH-insensitive luciferases and Asn in pH-sensitive luciferases) on the bioluminescence spectrum and pH-sensitivity using four model beetle luciferases cloned in our laboratories (12, 16–18): the green-emitting luciferase of the Japanese *Ragophthalmus ohbai* railroadworm (Rol); the red-emitting luciferase of the Brazilian *Phrixothrix hirtus* railroadworm (PhRE); the green-emitting luciferase of the Brazilian larval click beetle *Pyrearinus termitilluminans* (Pyt) and the green-emitting luciferase of the Japanese firefly *Pyrocoelia miyako* (Pml).

## MATERIALS AND METHODS

**Reagents.** Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), dithiothreitol (DTT), D-luciferin (sodium salt), guanidine chloride, ampicillin (Wako Pure Chemicals; Osaka, Japan; Sigma; St. Louis, MO); coenzyme-A (CoA) and adenosine triphosphate (ATP) (Oriental Yeast Co; Osaka, Japan; Sigma; St. Louis, MO); restriction enzymes and Taq polymerase (Nippon Gene; Toyama, Japan; New England Biolabs); ABI PRISM Dye terminator Cycle Sequencing kit (Perkin-Elmer; Foster City, CA) and Thermo sequenase Cy 5.5 dye terminator sequencing kit (Amersham Pharmacia, Tokyo, Japan). QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

**cDNAs.** The cDNAs for *Pyrearinus termitilluminans* (Pyt), *Phrixothrix hirtus* (PhRE), *P. vivianii* (PxGR), *Ragophthalmus ohbai* (Rol), and *Pyrocoelia miyako* (Pml) luciferases were previously cloned in our laboratories (12, 16–18).

**Site-directed mutagenesis.** Site directed mutagenesis was performed using a Stratagene mutagenesis kit (Catalog 200518). The plasmids containing the luciferases cDNAs were amplified using *Pfu* turbo polymerase and 2 complementary primers containing the desired mutation, using a thermal cycler (1 cycle 95°C; 12 cycles 95°C, 30 s; 55°C, 1 min and 68°C 12 min). After amplification, mutated plasmids containing staggered nicks were generated. The products were treated with *DpnI* in order to digest nonmutated parental plasmids, and used directly to transform *E. coli* XL-Blue 1 cells. The following primers and their respective reverse complements were used: (Pyt T226N) 5'CCCAGAGTTGGAAACCAACTTATTC3'; (PhRE T226N) 5'GTATACTATAAGCTTCGTCCATAGC3'; (Pml C259V) 5'TAC TTA ACG GTT GGA TTT CGT3'; (Pml N230S) 5'GTG TTT GGT AGT CAA ATT ATT3'; (RoL V255C) 5'GTC TTA TTT TAT ATG CGG GCT TAG AGT TG3'; (RoL T226S) 5'TTA TTC GGC TCA AGA ACT ATT3'; (RoL T226N) 5'CCC TTA TTC GGC AAT AGA ACT ATT CC3'; (RoL T226H) 5'CCC TTA TTC GGC CAC AGA ACT ATT CC3'; (RoL T226E) 5'CCC TTA TTC GGC GAG AGA ACT ATT CC3'; (RoL T226V) 5'CCC TTA TTC GGC GTA AGA ACT ATT CC3'; (RoL T226F) 5'CCC TTA TTC GGC TTT AGA ACT ATT CC3'.

**Sequencing.** The mutants were sequenced by dideoxy chain termination method (30) using dye-labeled terminator kit specifically developed for the ABI PRISM 377 automatic sequencer (Perkin-Elmer; Foster City, CA) or Thermo sequenase Cy5.5 Dye Terminator Cycle Sequencing kit specifically developed for the Gene rapid Amersham Pharmacia Biotech sequencer (Tokyo, Japan). The following primers were designed to sequence the mutations: (PhRE T226N) CCATCTATGGTAATCGTATTGCTCC; (Pyt T226N) GCACAA-CAATACGATTCTCG; (Pml) 5'TCA TCG GGA TCT ACT GGA TTA3'; (RoL) 5'TCC TCA TCG GGA ACA ACC GGG3'.

**Screening of color mutants.** Luciferase mutants were screened for BL intensity by photodetection (31). Screening for color mutants was done using a spectrofluorometric technique. Transformant colonies were randomly selected, isolated and replated on an area of 1 cm<sup>2</sup> over LB/Amp plates and grown overnight at 37°C. Replicas were obtained on nitrocellulose filters which were then transferred to LB/Amp/IPTG dishes for induction during 24 h at 20°C. Each colony containing lane was cut with a razor blade, exposed to drops of 1 mM luciferin (0.1 M sodium citrate pH 5.0) solution and stuck to a microscope slide placed in front of the spectrofluorometer window to obtain the bioluminescence spectrum. Screening for very weak bioluminescence intensity was done using a cooled-CCD camera system (Atto Co., Tokyo, Japan) (13).

**Extraction of luciferases.** For luciferase preparations, 200–1000 mL of culture were grown at 37°C up to OD<sub>600</sub> = 0.3–0.5 and then induced with 1 mM IPTG at 30–37°C until OD<sub>600</sub> = 1.0 for Pml and Rol luciferases and at 20°C until OD<sub>600</sub> = 1.7–1.9 for other luciferases. Cells were harvested by centrifugation at 10,000 rpm for 15 min and resuspended in extraction buffer (0.1 M sodium phosphate buffer, 1 mM EDTA, 1 mM DTT and 1% Triton X-100, pH 7.5), sonicated 6 times with 10s pulses and centrifuged at 15,000g for 15 min at 4°C. In some cases the supernatant was precipitated by ammonium sulfate and the proteins that precipitated between 55–70% were resuspended in extraction buffer and stored at –20°C.

**Measurement of luminescence intensities.** Bioluminescence intensities were measured in a Luminescencer AB-2000 luminometer (Atto; Tokyo, Japan) and in a homemade Hastings photometer (32). The assays were performed by mixing 10  $\mu$ L of crude extract and 100  $\mu$ L of assay solution (0.5 mM luciferin, 2 mM ATP, 4 mM MgSO<sub>4</sub> in 0.1 M Tris-HCl, pH 8.0). Light intensities were estimated by integration during 20 s and by peak height.

**Bioluminescence spectra.** Bioluminescence spectra were recorded using a Fluoromax Spex and a Hitachi F4500 spectrofluorometers according to Viviani *et al.* (16, 17). In both cases the emission spectra were autocorrected for instrument photosensitivity. For the *in vitro* BL spectra of PhRE and Pyt luciferases and their mutants, 50–100  $\mu$ L of crude extracts were mixed with 900–950  $\mu$ L of assay solution (0.5 mM luciferin, 2 mM ATP, 4 mM MgSO<sub>4</sub>, 0.5 mM CoA and 1% Triton X-100 in 0.1 M Tris-HCl, pH 8.0). For Rol and Pml luciferases and their mutants, the bioluminescence spectra were recorded after mixing 0.5 mL of luciferase solution to 0.5 mL of luciferin solution (2 mM ATP, 0.5 mM D-luciferin and 4 mM MgSO<sub>4</sub> in 0.1 M sodium phosphate buffer pH 8.0). CoA and Triton X-100 were used for luciferases displaying flash-like kinetics in order to stabilize and increase the emission, and have no effect on the bioluminescence spectra shape. The effect of pH was assayed 0.1 M phosphate buffer, pH 6–8.

## RESULTS AND DISCUSSION

Previously we have shown that click-beetle and railroadworm luciferases bioluminescence spectra are not affected by pH in contrast to firefly luciferases (28). Some of these conserved residues within each group, which differ between these two groups of luciferases could determine pH-sensitivity and affect the excited

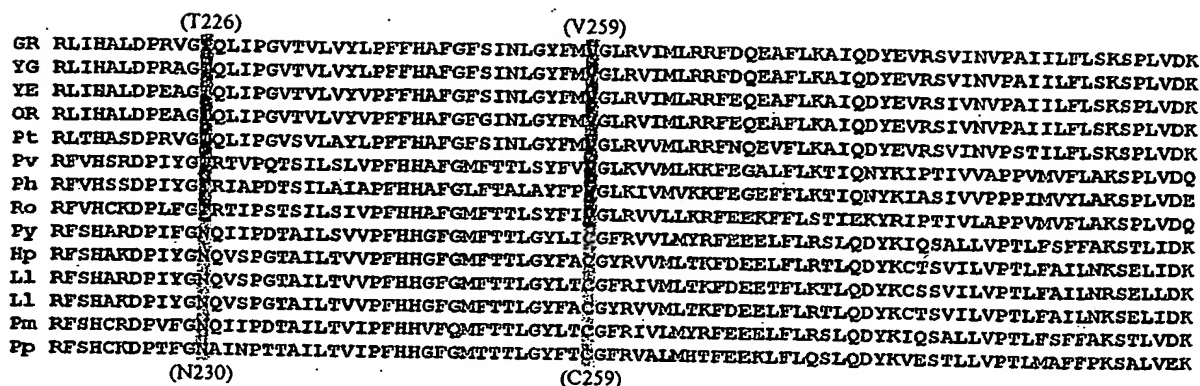


FIG. 1. Sequence alignment of the region between residues 200–300 of beetle luciferases showing residues Thr226 and V255. GR, YG, YE, and OR (*Pyrophorus plagiophthalmus* green, yellow-green, yellow, orange); Pt (*Pyrearinus termitilluminans*); Pv (*Phrixothrix viviani* green); Ro (*Ragophthalmus ohbai*); Py (*Photinus pyralis*); Pm (*Pyrocoelia miyako*); Hp (*Hotaria parvula*); Ll (*Luciola lateralis*); Pp (*Photuris pensilvanica*).

state of oxyluciferin (17). In this study, we mainly focus on two particular residues, Thr-226 and Val-255 in pH-insensitive luciferases, and the corresponding residues Asn-230 and Cys259 in *Pyrocoelia miyako* (Pm),

a pH-sensitive luciferase (Fig. 1). Table 1 shows the spectra of wild type (PmL and RoL) and mutant luciferases (PmL-C259V and RoL-V255C) under the various pH conditions. Cysteine residue was our first tar-

TABLE 1  
Comparison of Bioluminescence Spectra, Luciferin  $K_M$ s and Bioluminescence Intensity Half-Life Times for Wild-Type and Mutant Luciferases

Luciferase	$\lambda_{max}$ (nm)*			Half-bandwidth (nm)			$K_M$ (luciferin) ( $\mu$ M)†	Half-life (s)
	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8		
Wild-types								
RoL	548	548	548	73	76	76	1300	110
Pyt†	536	536	534	78		73	64**	36
PhRE‡	622		622	62		53	20**	5
PvGR	548	548	548	75		70.5	150**	126
PmL	612	562	554	91	76	76	10	5
Mutants								
PmL C259V	608	559	556	102	95	84		
PmL N230S	616	616	606	56	56	73		
RoL V255C	550	549	548	76	75	77		
Pyt T226N‡	541		546	67		77		
PhRE T226N‡			611			57		36
RoL T226 mutants								
RoL T226S		564	565		83	83		
RoL T226N	587	582	582	72	66	69	820	135
RoL T226H			584			74	110	60
RoL T226E			586			71		
RoL T226V			588			55		
RoL T226F			590			75	450	

\* Spectra were recorded after mixing 0.5 ml of luciferase solution to 0.5 ml of luciferin solution (2 mM ATP, 0.5 mM D-luciferin and 4 mM  $MgSO_4$  in 0.1 M sodium phosphate buffer, pH 6–8). Emission spectra were determined using a Hitachi F4500 with the excitation lamp shut down.

† 50  $\mu$ l of 4 mM ATP solution (8 mM  $MgSO_4$ , 0.1 M Tris-HCl buffer pH 8.0) was injected to 50  $\mu$ l crude extract diluted 10 times in 0.1 M Tris-HCl buffer, pH 8.0, containing luciferin (0.05–1 mM). The activity levels were measured using a Luminescencer AB-2000 (Atto; Tokyo, Japan).

‡ Bioluminescence spectra were recorded after mixing 50  $\mu$ l of luciferase crude extract and 950  $\mu$ l of luciferin solution (0.5 mM D-luciferin, 2 mM ATP, 4 mM  $MgSO_4$ , 0.5 mM CoA, 1% Triton X-100 in 0.1 M Tris-HCl, pH 8.0, or 0.1 M phosphate buffer, pH 6–8). Emission spectra were measured using a Spex Fluoromax spectrofluorometer.

\*\*  $K_M$  values from Viviani *et al.* (17).

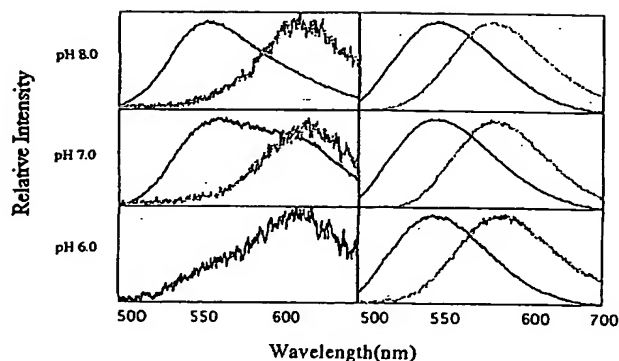


FIG. 2. Effect of pH on the bioluminescence spectra of wild-type (bold line) and mutant (light line) luciferases: (left) *Pyrocoelia miyako* wild-type and N230S mutant luciferases; (right) *Ragophthalmus ohbai* wild-type and T226N mutant luciferases. These spectra were autocorrected for the equipment photosensitivity.

get because it is a basic residue and early chemical modification studies have suggested that a cysteine could be the basic residue assisting oxyluciferin enolization to give rise to yellow-green bioluminescence (33). Both PmL-C259V and Rol-V255C had no considerable effect on the bioluminescence spectra nor their pH-sensitivity, indicating that these residues are not important for bioluminescence determination.

Table 1 shows the values of maximum wavelength and half-bandwidth of RoL, *Pyrearinus termitilluminans* luciferase (Pyt), *Phrixothrix hirtus* red light emitting luciferase (PhRE), and Thr-226 mutants. The substitution of Thr-226 by Asn, which is present in firefly luciferases, resulted in a 35 nm red shift on the bioluminescence spectrum of RoL and 12 nm in Pyt whereas T226N mutant in the red-light-emitting luciferase of PhRE showed a 10 nm blue shift. Interestingly, the half-bandwidth in these mutant luciferases was almost unchanged upon the substitution and the spectra of mutant luciferases shifted uniformly. In the firefly luciferase the reverse substitution, i.e., N230S, resulted in a typical red-mutant (Fig. 2). These results suggest that Thr226 is a key residue located close or within the active-site of both groups of beetle luciferases, interacting with excited oxyluciferin or with another residue important for keeping the active-site structure. Therefore, we made several mutants for Thr-226 to investigate the role of this residue in bioluminescence spectra (Fig. 3). Table 1 shows the comparison of the maximum wavelength, half-bandwidth and  $K_M$  values of Rol and Rol Thr-226 mutant luciferases. The red shift in these mutants varied from 17 nm for Ser 226 mutant to 42 nm for Phe226 mutant. However, these mutants did not show pH-sensitivity (Fig. 3) and their spectra shifted uniformly with small changes ( $\pm 20$  nm) in the half-bandwidth in relation to that of the wild-type luciferases. There was not a clear relationship between the physical-chemical properties of the substituted res-

idues and the magnitude of the red shift. However, the most drastic shifts were observed for Phe and Val, which are the most hydrophobic substitutions, and in addition Phe has the largest molecular volume. On the other hand, the conservative substitution T226S displayed the lowest red-shift among all mutants, suggesting a functional role for the hydroxyl group. Since the original residue Thr-226 is polar and could potentially form hydrogen bonds with other residues, it is possible that such substitutions result in the disruption of a structurally important polar interaction, and those substitutions with high steric hindrance and hydrophobic properties play an antagonistic effect resulting in the largest shifts.

The  $K_M$  values measured for Rol luciferase and its mutants were much higher than for other luciferases measured under the same condition. Surprisingly, the value for the wild-type Rol luciferase was higher than

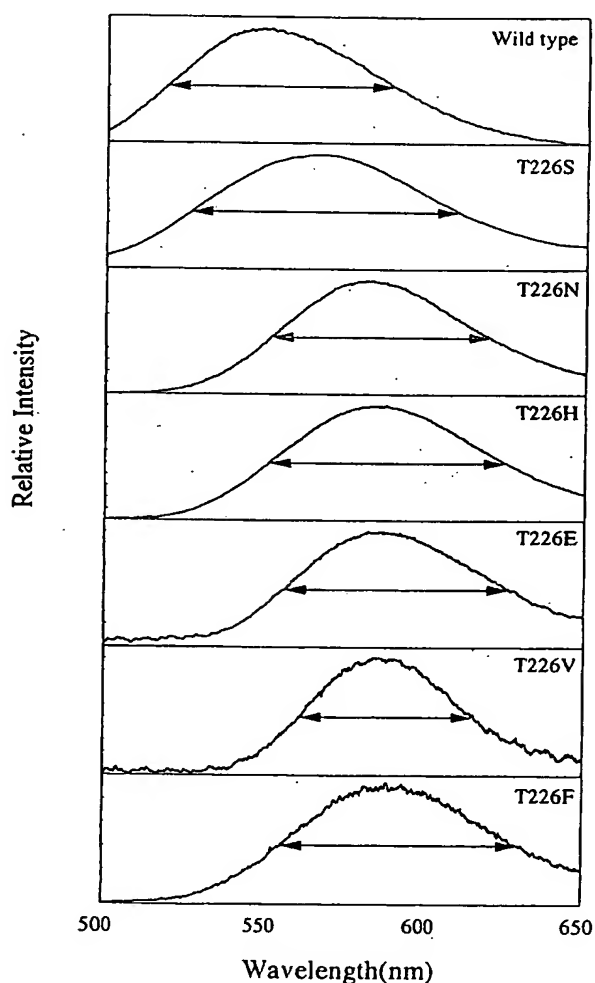


FIG. 3. Effect of T226 substitutions on *Ragophthalmus ohbai* luciferase bioluminescence spectra. These spectra were autocorrected for the equipment photosensitivity.

those for its mutants. As expected, luciferases with higher  $K_M$  values also displayed higher decay rates (Table 1). Higher substrate affinities also mean tightly bound products slowing the turnover rate and thus increasing the decay rates, whereas lower affinities should be reflected in higher turnover. Previous data on click-beetle and firefly luciferases suggested that lower  $K_M$  values relate with shorter wavelengths (34). Our results with railroadworm luciferases showed the opposite. In our opinion  $K_M$  values are not necessarily related with the position of the bioluminescence spectra peak but rather with the half-bandwidth since the latter reflect the degree of vibrational freedom of the emitter.

Chemical mechanisms for the color differences have been proposed, based on the presence of basic residue assisting oxyluciferin tautomerization (the keto form emits red light and the enolate emits green light) (6), on the polarity of the oxyluciferin binding site (5) and the active-site conformation restricting the degree of oxyluciferin thiazoline rings rotation (7). In firefly luciferases, the spectra of the wild-type and mutant luciferases are usually broad and contain two peaks, representing the contribution of two emitting species: the enolic and ketonic forms of excited oxyluciferin (35). The ratio between these emitters can be shifted by pH, temperature, mutations and by the luciferase itself (Fig. 2). These data suggest the color differences in firefly luciferases might be explained by changes in the ratio between the two emitting species and by environmental perturbations around these two emitters. However, the spectra of the wild type pH-insensitive beetle luciferases are narrower and monotonic suggesting emission by a single light emitting species. The spectra of their mutants shifted uniformly to longer wavelengths (shorter in the case of PhRE), suggesting that distinct bioluminescence colors could be achieved through small environmental perturbations such as the polarizability around a single emitting species. Furthermore, until present we never obtained a real red-mutant with pH-insensitive luciferases either by site-directed and random mutagenesis, such as in the case of firefly luciferases. Thus, the basic mechanism for the color determination between pH-sensitive and pH-insensitive luciferases is not identical. In our opinion the active-site core in pH-insensitive luciferases is rigid (looser in the green emitting ones and tighter in the red emitting one) and much less flexible than that of pH-sensitive luciferases.

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## REFERENCES

1. Biggley, W., Lloyd, H., J. E., and Seliger, H. H. (1967) Spectral distribution of firefly light. *J. Gen. Physiol.* 50, 1681-1692.
2. Colepicolo, N. P., Costa, C., and Bechara, E. J. H. (1986) Brazilian species of elaterid luminescent beetles. Luciferin identification and bioluminescence spectra. *Insect Biochem.* 16, 803-810.
3. Viviani, V. R., and Bechara, E. J. H. (1993) Biophysical and biochemical aspects of phengodid bioluminescence. *Photochem. Photobiol.* 58, 615-622.
4. Wood, K. V. (1995) The chemical mechanism and evolutionary development of beetle bioluminescence. *Photochem. Photobiol.* 62, 662-673.
5. DeLuca, M. (1969) Hydrophobic nature of the active site of firefly luciferase. *Biochemistry* 8, 160-166.
6. White, E. H., and Branchini, B. (1975) Modification of firefly luciferase with a luciferin analog. A red light producing enzyme. *J. Am. Chem. Soc.* 97, 1243-1245.
7. McCapra, F., Gilfoyle, D. J., Young, D. W., Church, N. J., and Spencer, P. (1994) The chemical origin of color differences in beetle bioluminescence. *In Bioluminescence and Chemiluminescence: Fundamental and Applied Aspects* (Campbell, A. K., Kricka, L. J., and Stanley, P. E., Eds.), pp. 387-391. Wiley, Chichester, UK.
8. De Wet, J. R., Wood, K. V., Helinsky, D. R., and DeLuca, M. (1985) Cloning of firefly luciferase cDNA and expression of active luciferase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 82, 7870-7873.
9. Tatsumi, H., Masuda, T., Kajiyama, N., and Nakano, E. (1989) Luciferase cDNA from Japanese firefly *Luciola cruciata*: Cloning, structure and expression in *E. coli*. *J. Biolum. Chemilum.* 3, 75-78.
10. Tatsumi, H., Kajiyama, N., and Nakano, E. (1992) Molecular cloning and expression in *E. coli* of a cDNA encoding luciferase of a firefly *Luciola lateralis*. *Biochim. Biophys. Acta* 1131, 161-165.
11. Devine, J. H., Kutuzova, G. D., Green, V. A., Ugarova, N. N., and Baldwin, T. O. (1993) Luciferase from the east European firefly *Luciola mingrelia* cloning and nucleotide sequence of cDNA, overexpression in *E. coli* and purification of the enzyme. *Biochem. Biophys. Acta* 1173, 121-132.
12. Ohmiya, Y., Ohba, N., Toh, H., and Tsuji, F. I. (1995) Cloning, expression and sequence analysis of cDNA for the luciferase from the Japanese fireflies, *Pyrocoelia miyako* and *Hotaria parvula*. *Photochem. Photobiol.* 62, 309-313.
13. Sala-Newby, G. B., Thomson, C. M., and Campbell, A. K. (1996) Sequence and biochemical similarities between the luciferases of the glow-worm *Lampyrus noctiluca* and the firefly *Photinus pyralis*. *Biochem. J.* 313, 761-767.
14. Li Ye, Buck L. M., Scaeffler, H. J., and Leach, F. R. (1997) Cloning and sequencing of a cDNA for firefly luciferase from *Photuris pennsylvanica*. *Biochim. Biophys. Acta* 1339, 39-52.
15. Wood, K. V., Lam, Y. A., Seliger, H. H., and McElroy, W. D. (1989) Complementary DNA coding click beetle luciferases can elicit bioluminescence of different colors. *Science* 244, 700-702.
16. Viviani, V. R., Silva, A. C. R., Perez, G. L. O., Santelli, R. V., Bechara, E. J. H., and Reinach, F. C. (1999) Cloning and molecular characterization of the cDNA for the Brazilian larval click-beetle *Pyrearinus termitilluminans* luciferase. *Photochem. Photobiol.* 70, 254-260.
17. Viviani, V. R., Bechara, E. J. H., and Ohmiya, Y. (1999) Cloning, sequence analysis, and expression of active *Phrixothrix* railroadworms luciferases: Relationship between bioluminescence spectra and primary structures. *Biochemistry* 38, 8271-8279.
18. Sumiya, M., Viviani, V. R., Ohba, N., and Ohmiya, Y. (1998) Cloning and expression of a luciferase from the Japanese lumi-

- nous beetle *Ragophthalmus ohbai*. In *Bioluminescence and Chemiluminescence. Proceeding of 10th International Symposium on Bioluminescence and Chemiluminescence* (Roda, A., Pazzagli, M., Kricka, L. J., and Stanley, P. E., Eds.), pp. 433–436, Bologna, Italy.
19. Wood, K. V. (1990) Luc genes: Introduction of colors into bioluminescence assays. *J. Biol. Chemilum.* 5, 107–114.
  20. Kajiya, N., and Nakano, E. (1991) Isolation and characterization of mutants of firefly luciferase which produce different colors of light. *Protein Eng.* 4, 691–693.
  21. Ohmiya, Y., Hirano, T., and Ohashi, M. (1996) The structural origin of the color differences in the bioluminescence of firefly luciferase. *FEBS Lett.* 384, 83–86.
  22. Mamaev, S. V., Laikhte, A. L. R., Arslan, T., and Hecht, S. M. (1996) Firefly luciferase: Alteration of the color of emitted light resulting from substitutions at position 286. *J. Am. Chem. Soc.* 118, 7243–7244.
  23. Ueda, H., Yamanouchi, H. i., Kitayama, A., Inoue, K., Hirano, T., Suzuki, E., Nagamune, T., and Ohmiya, Y. (1996) His-433 as a key residue for the color difference in firefly luciferase *Hotaria parvula*. In *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons. Proceeding of 9th International Symposium* (Hastings, J. W., Kricka, L. J., and Stanley, P. E., Eds.), pp. 216–219. John Wiley, Chichester, UK.
  24. Branchini, B. R., Magyar, R. A., Murtishaw, M. H., Anderson, S. M., and Zimmer, M. (1998) Site-directed mutagenesis of Histidine 245 in firefly luciferase: A proposed model of the active site. *Biochemistry* 37, 15311–15319.
  25. Conti, E., Franks, N. P., and Brick, P. (1996) Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes. *Structure* 4, 287–298.
  26. Branchini, B. R., Magyar, R. A., Murtishaw, M. H., Anderson, S. M., Helgersson, C., and Zimmer, M. (1999) Site-directed mutagenesis of firefly luciferase active-site amino-acids: Proposed model for bioluminescence color. *Biochemistry* 38, 13223–13230.
  27. Seliger, H. H. and McElroy, W. D. (1964) The colors of firefly bioluminescence: Enzyme configuration and species-specificity. *Proc. Natl. Acad. Sci. USA* 52, 75–81.
  28. Viviani, V. R., and Bechara, E. J. H. (1995) Bioluminescence of Brazilian fireflies (Coleoptera: Lampyridae): Spectral distribution and pH effect on luciferase-elicited colors. Comparison with elaterid and phengodid luciferases. *Photochem. Photobiol.* 62, 490–495.
  29. Viviani, V. R., and Ohmiya, Y. (2000) Bioluminescence color determinants of *Phrixothrix* railroadworm luciferases: Chimeric luciferases, site-directed mutagenesis of Arg 215 and guanidine effect. *Photochem. Photobiol.* 72, 267–271.
  30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning, a Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY.
  31. Wood, K. V., and DeLuca, M. (1987) Photographic detection of luminescence in *E. coli* containing the gene of firefly luciferase. *Anal. Biochem.* 161, 501–507.
  32. Mitchell, G., and Hastings, J. W. (1971) A stable inexpensive solid state photomultiplier photometer. *Anal. Biochem.* 39, 243–250.
  33. Alter, S. C., and DeLuca, M. (1986) The sulphhydryls of firefly luciferase are not essential for activity. *Biochemistry* 25, 1599–1605.
  34. Kutuzova, D. G., Hannah, R. R., and Wood, K. V. (1996) Bioluminescence color variation and kinetic behavior relationships among beetle luciferases. In *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons. Proceeding of 9th International Symposium* (Hastings, J. W., Kricka, L. J., and Stanley, P. E., Eds.), pp. 248–252. Wiley, Chichester, UK.
  35. White, E. H., Rapapor, E., Seliger, H. H., and Hopkins, T. A. (1971) The chemi- and bioluminescence of firefly luciferin: Efficient chemical production of electronically excited states. *Bioorg. Chem.* 1, 92–122.

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